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Enhanced Defense against Pneumocystis carinii Mediated by a Novel Dectin-1 Receptor Fc Fusion Protein

Rekha R. Rapaka,*† Eric S. Goetzman,* Mingquan Zheng,* Jerry Vockley,* Laura McKinley,* Jay K. Kolls,*† and Chad Steele2*†

Pneumocystis carinii (PC) pneumonia is a leading opportunistic infection found among HIV-infected individuals worldwide. Although CD4+ T cell deficiency clearly correlates with susceptibility to PC pneumonia, murine models of disease indicate that PC-directed Abs may prevent infection and/or inhibit growth of existing PC within the lungs. Recognition of PC by alveolar macrophages involves the β-glucan receptor Dectin-1 and macrophage effector function against PC is enhanced by Abs derived from PC-vaccinated hosts. We developed a fusion protein consisting of the extracellular domain of Dectin-1 linked to the Fc portion of murine IgG1, which we hypothesized would enhance host recognition and opsonic phagocytosis of PC. The recombinant protein, Dectin-Fc, is dimeric and the Ag recognition site identifies portion of murine IgG1, which we hypothesized would enhance host recognition and phagocytosis of PC. The recombinant protein, Dectin-Fc, is dimeric and the Ag recognition site identifies β-1,3 glucan linkages specifically and with high affinity ($K_D = 2.03 \times 10^{-7}$ M). Dectin-Fc enhances RAW264.7 macrophage recognition of the β-glucan containing particulate zymosan in an FcγRII- and FcγRIII-dependent manner and preopsonization of PC organisms with Dectin-Fc increased alveolar and peritoneal macrophage-dependent killing of PC. SCID mice treated with a replication incompetent adenoviral vector expressing Dectin-Fc had attenuated growth of PC within the lungs, overall decreased PC lung burden, and diminished correlates of PC-related lung damage relative to SCID mice receiving a control vector. These findings demonstrate that targeting PC β-glucan with Dectin-Fc enhances host recognition and clearance of PC in the absence of B and T cells, and suggest that FcγR-based targeting of PC, via cell wall carbohydrate recognition, may promote resistance against PC pneumonia in the immunodeficient host. The Journal of Immunology, 2007, 178: 3702–3712.

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3 Abbreviations used in this paper: PC, Pneumocystis carinii; HAART, highly active antiretroviral therapy; TMB, tetramethylbenzidine; MFI, mean fluorescence intensity; BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; CRD, carbohydrate recognition domain; PAMP, pathogen-associated molecular pattern; SP-D, surfactant protein D; CH, constant heavy.

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PC organisms to existing chemotherapeutic strategies and additionally underscores the need for alternate, immune-based strategies against PC infection.

Murine models of PC infection, using the host specific pathogen P. carinii f. sp. muris, have demonstrated the potential utility of Ab-based immunity in host defense against PC. CD4+ T cell deficiency or blockade of CD40L renders mice susceptible to PC and is coincident with diminished IgG responses against PC (8, 9). μMT knockout mice, deficient in B cells, are also highly susceptible to PC pneumonia and signify the critical nature of B cells and their effectors in PC resistance (10). Adoptive transfer of serum from PC-vaccinated mice or mAbs directed against PC Ags can prevent the establishment of infection upon challenge (11, 12) or inhibit progression of existing PC pneumonia in early through late stages of disease (13). Interestingly, mice challenged with PC and thereafter depleted of CD4+ T cells have persistent anti-PC IgG and are able to resist infection given a subsequent exposure, suggesting that Ab-based immunity may be sufficient for preservation of anti-PC resistance in the setting of CD4+ T cell deficiency (14). PC-directed Abs likely function through multiple mechanisms to increase host resistance to infection and have been shown to significantly potentiate alveolar macrophage anti-PC effector function in vitro (15–17). Though recent studies suggest that PC-specific IgG is not absolutely required for host resistance, FcγR-deficient mice clear PC organisms with delayed kinetics (18). These observations suggest that PC-specific Abs may couple with FcγR-bearing cells to modulate aspects of host recognition and clearance of PC and illustrate the potential of Ab-based strategies against PC in the setting of immunodeficiency.

The specific Ags involved in Ab-based recognition and degradation of PC, identifying either cystic and/or trophozoite forms of the organism, are unclear. PC protein Ags under investigation as vaccine targets, such as the major surface glycoprotein complex...
and the surface-associated protease Kexin, exhibit interspecies- and intraspecies-specific variation (17, 19, 20), which may complicate potential vaccination strategies. Conserved-pathogen-associated molecular patterns (PAMPs) of PC identified by innate immune cells include the major carbohydrates of the fungal cellular wall, β-glucan, and mannan. It is unknown whether these carbohydrate Ags could be effective targets of Ab-based responses. We have previously shown that the β-glucan receptor Dectin-1 is involved in alveolar macrophage recognition, nonopsonic phagocytosis, and killing of PC in vitro (16). RAW 264.7 macrophages overexpressing Dectin-1 bound PC organisms at higher levels than control cells, suggesting a critical, and possibly independent, role of this receptor in PC identification. In this study, we report on the potential of a recombinant protein, consisting of the extracellular domain of Dectin-1 fused to the murine IgG1 hinge through constant heavy (CH)2 and CH3 domains, as a novel strategy for the identification, specific targeting, and degradation of PC organisms.

Materials and Methods

Mice

Male C57BL/6 mice, 6–8 wk of age, were purchased from the National Cancer Institute, National Institutes of Health. Male B6.scid mice, 6–8 wk of age, were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free environment in microisolator cages within an American Association for Laboratory Animal Science-certified animal facility in the Rangos Research Center at the Children’s Hospital of Pittsburgh. Animal studies were reviewed and approved by the Children’s Hospital of Pittsburgh Animal Research and Care Committee.

DNA constructs and adenoviral vectors

The design of the Dectin-Fc recombinant gene construct was modeled after the TNFR-IgG H chain, described by Peppel et al. (21). A PCR 3.1 plasmid containing the full-length murine Dectin-1 receptor, provided by G. Brown (University of Cape Town, Cape Town, South Africa), and the pACPK2 plasmid containing the TNFR extracellular domain linked to murine IgG1 H chain (22) served as PCR templates. The cDNA encoding the extracellular domain of the Dectin-1 receptor, consisting of amino acids 69–244 (23), was amplified by PCR with oligonucleotide primers ggtaccga CGA GTCTTCTTCCAGG (corresponding to an engineered KpnI site, a frame spacer, and the 5′ end of the Dectin-1 receptor extracellular domain) and ggtaccgaccaacag CGA CACATAATCGGG (amplified as a control surface and the 3′ end of the Dectin-1 receptor). The PCR product encoding the CH2–CH3 domain was amplified with primers ggtaccgagactgcactggtagctgcccaacag (corresponding to the hexapeptide linker with thrombin cleavage site and the 3′ end of the Dectin-1 receptor). The PCR product encoding CH2–CH3 was digested with enzymes KpnI and EcoRI, and ligated into the multiple cloning site of pSecTag 2C mamalian expression vector (Invitrogen Life Technologies), containing the Igκ leader sequence facilitating protein secretion. To confirm the fusion gene product, dideoxy sequencing was performed. Ad-Dectin-Fc is an E1-E3 replication-deficient rAd5-based vector containing the entire pSecTag 2C-Dectin-Fc expression cassette. To generate Ad-Dectin-Fc, the Dectin-Fc expression cassette was amplified and inserted into the Invitrogen Gateway entry vector pENTR/D-TOPO, then shuttled in an exchange reaction using LR Clonase into pAd/CMV/V5 DEST (Invitrogen Life Technologies). The resultant plasmid was purified, digested with PstI to expose adenoviral ITR sequences, and then transfected into 911 cells supplying the E proteins required for adenovirus propagation. Viruses were purified and concentrated by either CsCl density gradient centrifugation or by membrane adsorption (Vivapure Adenopack; Sartorius), and titered by a plaque assay on 911 cells, as previously described (22, 24). The control AdLuciferase vector is identical but instead encodes the firefly luciferase gene and was obtained from the Pittsburgh Pre-Clinical Vector Core. The particle:PFU ratio was ~100:1 and virus stocks contained <0.01 ng/ml endotoxin as determined by the OQL-1000 Limulus lyase assay (BioWhittaker).

Production of Dectin-Fc and Western blot

For Dectin-Fc protein generation, 293 cells cultured in DMEM plus 10% FCS were transiently transfected with the pSecTag 2C-Dectin-Fc vector with Lipofectamine 2000 (Invitrogen Life Technologies). Supernatants were collected and cells were removed by centrifugation and passage of supernatant over a 0.2 μm low protein-binding filter. Supernatants were preserved at −80°C. For some experiments, Dectin-Fc-conditioned supernatant or purified protein was isolated on SDS-PAGE, transferred to nitrocellulose membranes, and blocked overnight with BSA (2% for tissue culture supernatant, 4–8% for mouse BAL, serum, or lung homogenate). Rat anti-murine-Dectin-1 (R&D Systems) followed by goat anti-rat IgG-AP (Santa Cruz Biotechnology), or goat anti-mouse IgG-AP (Bio-Rad), all at 1:2000 in blocking buffer, were used for immunodetection, and blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/NBT reagent (Bio-Rad).

β-glucan ELISA

Laminarin and mannan (Sigma-Aldrich) were each dissolved in PBS and seeded to a Nunc polysorp-treated 96-well plates at 0.025 mg/ml at 4°C for 48 h. As a negative control, β-1,3-glucan linkages of laminarin were hydrolyzed by pretreatment with laminaranase (β-1,3 endoglucosidase) isolated from Trichoderma sp. as per manufacturer’s protocol (Sigma-Aldrich) and similarly seeded at 0.025 mg/ml. The wells were washed, blocked for 2 h in PBS, 10% FBS, and 2.5% milk, then washed again. Dectin-Fc-conditioned supernatant or purified Dectin-Fc was dissolved in blocking buffer, serially diluted 1/2, and administered to wells in triplicate. After 2 h of incubation, wells were washed, and a 1/1000 dilution of goat-anti-mouse IgG-HRP was applied for 2 h. Wells were washed and developed with tetramethylbenzidine (TMB; BD Biosciences-BD Pharmingen) and the absorbance at 450 nm was measured and subtracted from baseline absorbance.

BLacore analysis

Real-time surface plasmon resonance experiments were performed on a BLAcore 3000 Instrument with CM5 sensor chips (BLAcore) and all interactions were studied at 25°C, N-hydroxy succinimide/N,N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride amine coupling was used to immobilize goat anti-mouse Abs (Molecular Probes) diluted 1/50 in 10 mM sodium acetate (pH 5) and empty reactive sites were quenched with 1 M ethanolamine. Affinity purified Dectin-Fc (100 μg/ml) was captured by injection at a flow rate of 5 μl/min (total 35 μl). Laminarin was dissolved in PBS and diluted to 100, 2, or 0.2 μm in running buffer (10 mM HEPES (pH 8.0), 150 mM NaCl, 0.002% Tween 20), injected by KINET and allowed to reach equilibrium, after which only running buffer was applied. Immobilized goat anti-mouse Abs served as a control surface and nonspecific binding of laminarin was subtracted from the surface plasmon resonance signal in the active flow cell. The association rate constant (ka) and dissociation rate constant (kd) were calculated and the dissociation constant was determined (Kd) using the BIAevaluation 3.1 software.

P. carinii f. sp. muris isolate

The PC inoculum was prepared as previously described (8, 24). Briefly, B6.scid mice with PC pneumonia were injected with a lethal dose of penobarbital and the lungs were aseptically removed and frozen in 1 ml of PBS at −80°C. Lungs were mechanically dissociated in sterile PBS, filtered through sterile gauze, and pelleted at 500 × g for 10 min at 4°C. The pellet was resuspended in sterile PBS and a 1/5 dilution was stained by a modified Giemsa stain (Diff-Quik; Baxter). The number of PC cysts was quantified microscopically and the inoculum concentration was adjusted to 2 × 106 cysts/ml. Gram stains were performed on the inoculum preparations to exclude contamination with bacteria. For in vitro studies, aliquots of the inoculum were stored at concentrations of 106 cysts/ml at −80°C until use.

Peritoneal and alveolar macrophage isolation

For peritoneal macrophage isolation, male C57BL/6 mice were administered 3% sterile thioglycolate i.p. After 3–5 days, mice were sacrificed and a peritoneal lavage was performed using 5 ml of PBS. The lavage fluid was centrifuged at 300 × g for 10 min, resuspended in RBC lysis buffer.
(Sigma-Aldrich) for 5 min, then washed, pelleted, and resuspended in RPMI 1640. Cell pellets were studied and enumerated using a hemacytometer. To isolate alveolar macrophages, male C57BL/6 mice were anesthetized with isoflurane and sacrificed via terminal exsanguination. With an intratracheal catheter, calcium and magnesium-free PBS was used to lavage lungs. A total of 10 ml was used per mouse in 0.5-ml increments with a 30 s dwell time. The lavage fluids were pooled and centrifuged at 300 × g for 10 min, and the cells were collected for the coculture assay. Cell preparations were generally >98% enriched for peritoneal or alveolar macrophages.

Zymosan association assay
To assess macrophage surface association with zymosan, 10⁴ RAW 264.7 cells were suspended in RPMI 1640 plus 10% FCS in 5-ml polystyrene tubes. Some groups were pretreated for 30 min with 5 μg of Zymo (provided by G. Brown, as described in Ref. 25), a mAb with specific blocking activity against the murine Dectin-1 receptor, and/or 2.5 μg of FcγRIII blocking Ab 2.4G2 (eBioscience), and maintained at 37°C. FITC-zymosan (Molecular Probes) was suspended in culture medium, sonicated, enumerated, and preopsonized with conditioned supernatant containing Dectin-Fc, control supernatant consisting of DMEM plus 10% FCS, or neat normal mouse serum, for 30 min at 4°C. Ten particles of preopsonized zymosan were administered per macrophage and allowed to incubate with macrophages at 37°C for 90 min. A total of 10,000 events were analyzed with a FACSArray flow cytometer for relative FITC intensity (BD Biosciences). Macrophages were distinguished from free zymosan by forward and side scatter profiles. Mean fluorescence intensity (MFI) was calculated by averaging all events across the macrophage live cell gate; fold changes were calculated by normalizing the observed MFI to the baseline MFI obtained from RAW cells incubated with zymosan preopsonized with control medium.

Staining of Pneumocystis organisms with Dectin-Fc
PC organisms were heat-fixed to glass slides and incubated with Dectin-Fc-conditioned medium or control medium. After primary incubation, organisms were extensively washed; bound Dectin-Fc was detected by incubating with Cy3-conjugated goat anti-mouse-IgG. Following additional washes, ProLong-mounting medium and coverslips were applied. Slides were examined using a Zeiss Axiosplan 2 upright fluorescence deconvolution microscope and images were captured using 3× Slidebook version 4.0 software.

Pneumocystis viability assay
Macrophages (10⁴/ml) suspended in a volume of 100 μl of RPMI 1640 medium containing FCS were cocultured in round-bottom 96-well plates with PC (2 × 10⁶ cysts/ml, 50 μl), yielding an effect on total PC organism ratio of 1:1 (estimated 1:10 cyst to trophozoite ratio). Before addition of PC, organisms were preopsonized with 50 μl of Dectin-Fc-conditioned supernatant/purified Dectin-Fc or 50 μl of DMEM plus 10% FCS. A viability control of PC incubated with control medium, consisting of DMEM plus 10% FCS, was included. The plates were spun at 2500 rpm to pellet PC organisms. The supernatants and cell pellets were collected and total RNA was isolated using TRIzol-LS reagent (Invitrogen Life Technologies). Viability of PC was assessed with real-time PCR measurement of PC rRNA copy number (GenBank accession number AF257179) and quantified against a standard curve of known copy number of PC rRNA as previously described (16). This method detects viable PC organisms as evidenced by loss of detectable PC rRNA in heat-killed organisms or those exposed to trimethoprim/sulfamethoxazole.

Adenoviral gene transfer and Pneumocystis challenge
SCID mice (6–8 wk) received adenovirus at 1 × 10⁹ PFU perorally (total volume 100 μl) under isoflurane analgesia. Three days later, mice were sacrificed at a disulfide-linked bivalent structure (Fig. 1B). Under reducing conditions, the molecular mass of the protein product was halved, demonstrating that the recombinant protein adopts a disulfide-linked bivalent structure (Fig. 1B). A schematic portrayal of the fusion protein itself is presented in Fig. 1C. As the murine hinge plus Fc domain is 60 kDa, the extracellular domain of dectin-1 is roughly 28 kDa, in accordance with a previous report describing the molecular structure of the receptor (23).

Analysis of Dectin-Fc Ag-binding site
To define the properties of the β-glucan molecular recognition site of Dectin-Fc, we assessed its ability to recognize laminarin, a ~7.7-kDa water soluble carbohydrate β-glucan polymer isolated from Laminaria digitata. Laminarin contains primarily β-1,3 glucan linkages with some β-1,6 glucan linkages, similar in ratio to the β-glucan linkages found in the cellular wall of Saccharomyces cerevisiae and PC (27–29). In a study where dectin-1 was expressed exogenously in the nonphagocytic NIH3T3 cell line, laminarin was identified as the most potent competitive antagonist of the Ag recognition site portrayed in Fig. 1. As the murine hinge plus Fc domain is 60 kDa, the extracellular domain of dectin-1 is roughly 28 kDa, in accordance with a previous report describing the molecular structure of the receptor (23).

Analysis of Dectin-Fc β-glucan linkages
We next determined the affinity of the Dectin-Fc CRD for β-glucan linkages with real-time surface plasmon resonance measurements (BLAcore). Affinity purified Dectin-Fc was captured with

BALF analyses
Fluid from the lower respiratory tract was obtained by bronchoalveolar lavage of mice anesthetized with i.p. pentobarbital as described previously (21). The first milliliter of BALF from SCID mice infected with PC was collected at 5, 14, and 28 days was processed at 300 × g and supernatant was stored at −80°C until use. BALF samples were studied for Dectin-Fc expression or for relative lactate dehydrogenase (LDH) activity as determined with an LDH colorimetric assay kit (Sigma-Aldrich) detecting the decrease of substrate NADH → NAD⁺. Absorbance was determined at 530 nm. The remaining cell pellet and an additional 9 ml of BALF was pooled and centrifuged at 800 × g for 10 min to collect cells and samples were cytospun onto slides, stained by modified Giemsa, and analyzed for leukocyte differential.

Statistical analysis
Data were analyzed using GraphPad statistical software. Comparisons between groups when data were normally distributed were made with the Student t test; comparisons among nonparametric data were made with the Mann-Whitney U test. The Wilcoxon rank-sum test was used when comparing experimental groups to a theoretical value. Significance was accepted at a value of p < 0.05.

Results
Characterization of Dectin-Fc protein reactivity and structure
A recombinant fusion gene consisting of the extracellular domain of the murine dectin-1 receptor (amino acids 69–244), a thrombin sensitive hexapeptide linker, and the murine IgG1 hinge and CH₂ through CH₃ domains, was constructed and inserted into the eukaryotic expression vector, pSecTag 2C, directing protein secretion (data not shown). The cloned portion of dectin-1 contains the entire, relatively compact, carbohydrate recognition domain (CRD) of the receptor, which includes two putative N-glycosylation sites. Western analysis of the supernatant generated from 293 transfectants revealed a 116-kDa product, reactive with both goat-anti-mouse IgG Abs (Fig. 1A) and anti-murine dectin-1 Ab (Fig. 1B). Under reducing conditions, the molecular mass of the protein product was halved, demonstrating that the recombinant protein adopts a disulfide-linked bivalent structure (Fig. 1B). A schematic portrayal of the fusion protein itself is presented in Fig. 1C. As the murine hinge plus Fc domain is 60 kDa, the extracellular domain of dectin-1 is roughly 28 kDa, in accordance with a previous report describing the molecular structure of the receptor (23).

Analysis of Dectin-Fc Ag-binding site
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We next determined the affinity of the Dectin-Fc CRD for β-glucan linkages with real-time surface plasmon resonance measurements (BLAcore). Affinity purified Dectin-Fc was captured with
immobilized goat-anti-mouse Abs; laminarin was applied at various doses (0.2, 2, and 100 μM) and studied for interactions with the Dectin-Fc Ag-binding site (Fig. 3). As expected, laminarin bound Dectin-Fc and binding was dose dependent. The rate of association ($k_a$) was 5.6 × 10^7 M⁻¹ s⁻¹ and the rate of dissociation ($k_d$) was 1.14 × 10⁻² s⁻¹, signifying that the stability of the complex slightly exceeds its capacity to form. An overall equilibrium dissociation constant ($K_d$) of 2.03 × 10⁻⁷ M was calculated, demonstrating high-affinity binding of Dectin-Fc to laminarin, similar in quality to various carbohydrate directed Abs or lectin receptors for their ligands (31–35).

**Dectin-Fc enhances macrophage recognition of zymosan**

To address the potential of Dectin-Fc to enhance macrophage recognition of particulate β-glucan, we evaluated whether Dectin-Fc could enhance RAW264.7 macrophage recognition of fungal PAMP-containing particulate zymosan. Zymosan is an insoluble cellular wall polysaccharide derived from *S. cerevisiae*, composed mostly of β-glucan and mannan (36). FITC-labeled zymosan was preopsonized with either Dectin-Fc-conditioned medium, control medium, or normal mouse serum and added to macrophages for 90 min, and macrophage-associated FITC fluorescence was determined by flow cytometry as a correlate of cellular association with zymosan. As demonstrated in Fig. 4A, we observed enhanced binding by macrophages of zymosan opsonized with normal mouse serum, which increases baseline binding of zymosan predominantly via CR3-based recognition (25). However, we observed even further enhancement of macrophage recognition when zymosan was instead preopsonized with Dectin-Fc (Fig. 4A). Analysis of the average macrophage MFI showed that recognition of zymosan by RAW cells was significantly inhibited by Dectin-1 receptor blocking Ab 2a11, as previously reported (25), and that Ab-mediated blockade of FcγRII and FcγRIII did not perturb FITC-zymosan association from baseline levels (Fig. 4B). Yet, Dectin-Fc enhanced the average cellular MFI by 27% from baseline levels (Fig. 4B), and the observed increased recognition of Dectin-Fc preopsonized zymosan was entirely lost upon blockade of macrophage FcγRII and FcγRIII (Fig. 4B). As murine IgG1 immune complexes signal primarily through FcγRII and FcγRIII (37), we conclude that Dectin-Fc-opsonized zymosan was recognized through binding at these receptors. Interestingly, when the Dectin-1 receptor on macrophages was blocked, recognition of Dectin-Fc-preopsonized zymosan was further enhanced (Fig. 4B).

Although the mechanism through which this occurs is unclear, it raises the possibility that immune-complex associated, multimerized FcRs generate stronger or more prolonged macrophage associations with zymosan relative to that which occurs under competitive binding with the Dectin-1 receptor. Thus, Dectin-Fc enhances recognition of zymosan by macrophages, and in the absence of cellular Dectin-1 receptor recognition, by promoting binding through FcγRII and FcγRIII.

**Dectin-Fc binds Pneumocystis organisms and enhances macrophage-dependent killing**

The cellular wall of the PC cyst consists of a thick electron-lucent layer composed predominantly of β-glucan, buried below a thin, electron-dense surface layer consisting mainly of mannan and glycoprotein (27, 38). Despite a seemingly shielded location, it is believed that PC β-glucan is sufficiently exposed to permit recognition by the innate immune system via Dectin-1 (16) and other pattern recognition receptors, such as the lactosylceramide-associated β-glucan receptor (39). Indeed, the specific interaction of PC β-glucan with these innate receptors has been demonstrated to influence patterns of cytokine and chemokine expression, such as the elicitation of the neutrophil chemoattractant MIP-2. By fluorescent deconvolution microscopy, we observed that Dectin-Fc bound to the surface of PC cysts (Fig. 5A). This observation demonstrates the accessibility of Dectin-Fc to β-glucan ligands on PC organisms.

As we observed specific binding of Dectin-Fc to PC organisms, we evaluated whether Dectin-Fc could enhance the recognition and degradation of Pneumocystis organisms by FcγRII/III-bearing...
cells. We studied decreases in absolute quantities of PC large rRNA copy numbers in the presence or absence of macrophages as a correlate of in vitro PC killing, a methodology validated by previous work (16, 40). PC organisms were isolated from infected murine lung homogenates and were preopsonized with Dectin-Fc, then administered to macrophages at a 1:1 PC organism to effector cell ratio for 24 h. In studies with thioglycolate-elicited peritoneal macrophages, preopsonization of PC organisms with Dectin-Fc diminished overall copy numbers by 3-fold relative to organisms preopsonized with control medium (Fig. 5B). When macrophages were pretreated with an Ab blocking activity at the Dectin-1 receptor, killing of Dectin-Fc preopsonized PC occurred to a similar degree. This observation suggested that Dectin-Fc promoted PC killing independent of native Dectin-1 recognition. Additional blockade of FcγRII and FcγRIII abrogated the killing effect, indicating that Dectin-Fc-dependent targeting of PC toward FcγRII and FcγRIII was responsible for the diminished PC rRNA signal.

Resident alveolar macrophages appeared to have a moderately higher level of effector activity against Dectin-Fc preopsonized PC compared with recruited peritoneal macrophages, decreasing PC rRNA copy numbers by nearly 10-fold relative to medium opsonized PC (Fig. 5, C and D). We hypothesize that differences between alveolar and elicited peritoneal macrophage populations in responses such as phagocytosis, reactive oxide species generation, and Ab-dependent killing may be responsible for the enhanced effector function against Dectin-Fc preopsonized PC by alveolar macrophages. We conclude from these studies that primary murine effector function against Dectin-Fc preopsonized PC is mediated via FcγRII and FcγRIII, and this occurs independently of dectin-1-based recognition.

**Dectin-Fc enhances clearance of Pneumocystis in SCID mice**

To study the effect of Dectin-Fc on PC infection in vivo, we developed a replication incompetent type 5 adenovirus, Ad-Dectin-Fc, containing the entire Dectin-Fc expression cassette, including an IgG leader sequence facilitating protein secretion upon cellular transduction. As IgG protein administered directly into the lungs is rapidly degraded (41), we considered adenoviral-based transgene delivery of Dectin-Fc as a more appropriate system to evaluate the effect of Dectin-Fc on this chronic, slow-growing infection. Systemic administration of adenoviral vectors encoding transgenes leads to high and prolonged levels of transgene protein in the lungs in the absence of virus (42) and mice deficient in CD4^+ T cells do not mount significant neutralizing Ab responses against adenoviruses (43), underscoring the suitability of gene transfer as an effective means for long-term expression of Dectin-Fc in the setting of immunodeficiency. SCID mice were treated with either control medium, serum, or Dectin-Fc-conditioned culture medium and administered to RAW 264.7 macrophages for 90 min. 2a11 and anti-CD16/32 Abs were preadministered to macrophages to block the dectin-1 receptor or FcγRII/FcγRIII, respectively. A total of 10,000 cells were collected and analyzed by flow cytometry for association with FITC particles. A, Histogram plot from zymosan association assay showing baseline fluorescence of macrophages (gray line), macrophages plus FITC-zymosan opsonized with control medium (dotted line), macrophages plus FITC-zymosan opsonized with serum (gray line, filled), and macrophages plus FITC-zymosan opsonized with Dectin-Fc (black line). B, Cumulative results from five separate experiments investigating the effects of Dectin-Fc on FITC-zymosan binding to RAW 264.7 macrophages, normalized to baseline MFI. For comparisons to the baseline condition, * represents a p < 0.05 and ** represents a p < 0.01 as calculated with the Wilcoxon rank-sum test after setting the baseline at a theoretical value of 1. For comparisons between Dectin-Fc and Dectin-Fc plus Fc block, ++ represents a p < 0.05 by Student’s t test.
Dectin-Fc was observed in the BALF, demonstrating the presence of the fusion protein within the mucosal surfaces and within compartments typical of murine IgG.

To assess the ability of Dectin-Fc to enhance host clearance of PC, SCID mice were treated with Ad-Dectin-Fc or Ad-luciferase i.v. and rested for 3 days, followed by intratracheal challenge with 2 × 10^5 PC cysts. Mice were then sacrificed at specific time points after challenge and studied for PC burden within the lungs by assessment of total copy numbers of PC large rRNA subunit (Fig. 6B). Despite equivalent cyst counts for the inoculum between studies, PC rRNA counts were significantly different and this may be due to the challenge of quantifying noncystic PC forms. In a first experiment, 14 days after PC challenge, an average of 25% of the original inoculum is present in the Ad-luciferase-treated mice while the Ad-Dectin-Fc-treated mice contained only an average of 6.9% of the original inoculum. In a second experiment, 28 days after PC infection, mice treated with Ad-luciferase contained a substantially higher quantity of PC in the lungs at an average of 549% of original inoculum relative to the Ad-Dectin-Fc-treated mice (averaging at 139% of the inoculum). A third study, performed where a single inoculum was administered and mice were analyzed at serial time points, confirmed the inhibition of the growth kinetics of the PC organism within the lungs of mice receiving a control vector compared with mice receiving Dectin-Fc (Fig. 6C). Hence, treatment of mice with Dectin-Fc before PC infection considerably diminished the growth of the organism within the lungs, contributing to the ability of the SCID host to prevent PC multiplication within the lung environment. The efficacy of Dectin-Fc to inhibit PC growth is most apparent at later stages of PC infection, as innate host clearance mechanisms lose their capacity to control PC replication within the lungs.

**Effect of Dectin-Fc on correlative markers of lung injury in Pneumocystis-infected SCID mice**

Although hyperinflammatory responses mediated by CD8⁺ T cells generate significant lung damage and dysfunction in the murine response to PC (45), SCID mice suffer lung damage at late stages of pneumonia as a consequence of uncontrolled PC replication and immunodysregulation (46, 47). We assessed the capacity of Dectin-Fc to limit correlates of pulmonary damage associated with PC infection by assessing the presence of intracellular enzymes in the alveolar fluid. BALF collected from the study described in Fig. 6C was analyzed over the time course of infection for LDH activity. We observed significantly decreased levels of BALF LDH in mice treated with Dectin-Fc, compared with control mice, even as early as 5 days after PC challenge, when there is low PC burden (Fig. 7A). As a significant portion of the PC inoculum is cleared soon after intratracheal instillation, as we (Fig. 6C) and others previously observed (14), it is possible that targeting of PC to myeloid cells via FcγR-dependent host recognition contributed to this phenotype. Twenty-eight days after PC challenge, when there is a significantly higher PC burden in the lungs of control mice relative to Dectin-Fc-treated mice, BALF LDH was nearly twice as high in control mice relative to mice receiving Dectin-Fc, suggesting that Dectin-Fc limited both PC burden and cellular damage associated with infection. Also by day 28 postchallenge, there were significant differences in the quantities of neutrophils observed in the BALF, with decreased levels in mice receiving Dectin-Fc (Fig. 7B). It has been shown that SCID mice recruit high numbers of neutrophils into the lungs at late stages of PC infection which is correlative with lung damage (46) and in humans with PC pneumonia, increased BALF neutrophil counts are associated with poor prognosis (48, 49). MIP-2, a neutrophil chemoattractant secreted
FIGURE 6. In vivo activity of Dectin-Fc in SCID mice challenged with PC. A. A replication incompetent Ad5 vector containing the entire pSecTag2C Dectin-Fc expression cassette was generated. SCID mice were i.v. treated with $1 \times 10^7$ PFU Ad-Dectin-Fc or control vector Ad-Luciferase; Dectin-Fc expression was assessed in various compartments at given time points after treatment. Individual mouse samples were studied for reactivity with mDectin-1 Abs at 116 kDa. B. Three days post-gene transfer ($n = 5/condition/time point$), mice received $2 \times 10^5$ cysts i.t. and at specific time points thereafter, lungs were analyzed for total PC large rRNA subunit copy numbers. Days 14 (top) and 28 (middle) are displayed. Boxes represent the interquartile range of data between the 25th and 75th percentiles and whiskers represent the upper and lower limits of the data. The median is represented by the dividing line within the box. Bottom, Average copy numbers of PC organisms within the lungs of individual SCID mice normalized to the original inoculum per time point, plotted over time. C. To confirm inferences on kinetic growth a single inoculum was administered to mice ($n = 5/condition/time point$), lungs were harvested at various time points, and total PC large rRNA copy numbers are displayed. D. Data pooled from both SCID mice studies, day 28 after PC challenge ($n = 10/time point/group$), with individual PC lung burdens expressed as a percentage of total inoculum. In all frames, * represents a $p$ value $<0.05$, and ** represents a $p$ value of $<0.01$ between mice receiving Ad-Luciferase (control) and Ad-Dectin-Fc (Dfc) by Mann-Whitney $U$ test.
modified lipopeptides that inhibit the fungal-specific enzyme β-1,3-glucan synthase, rapidly diminish PC organisms in rodent models of infection (60, 61). Given the abundance and apparent accessibility of PC β-glucan, as well as its potentially critical role in the fungal life cycle, we considered immune-based strategies targeting this defined cell wall component as a novel therapy for PC pneumonia.

As macrophages overexpressing Dectin-1 have enhanced recognition and uptake of PC organisms relative to normal macrophages, we hypothesized that the CRD of Dectin-1 might be a sufficient targeting structure for PC identification. Immunoprotective Abs against PC enhance effector function at the level of the myeloid cell and, as production of PC-specific IgG1 is most perturbed by murine CD4+ T cell dysfunction (62), we chose to couple the Dectin-1 CRD with the murine IgG1 Fc fragment to promote recognition and signaling through FcγRs. We expected that Dectin-Fc would function similarly to an Ab and would promote host recognition and clearance of PC organisms by enhancing the effector function of FcγR-bearing cells.

The recombinant protein, Dectin-Fc, demonstrated specific binding to laminarin, a β-glucan-containing glycosidic linkages analogous in stoichiometry to that observed in the PC cyst wall (27). Hydrolysis of β-1,3 linkages of laminarin entirely blocked Dectin-Fc recognition, indicating its specificity for these glycosidic bonds. Characterization of the Dectin-Fc CRD with surface plasmon resonance measurements revealed a $K_D$ of $2.03 \times 10^{-7}$ M for laminarin, suggestive of high-affinity interaction. The observed affinity of Dectin-Fc for β-glucan is similar, if not somewhat higher, than that observed for lectin receptors; this may be attributable to the dimeric nature of the fusion protein. For example, the interaction of SIGN-R, a major mannose receptor on peritoneal macrophages, with terminal mannose epitopes is characterized by a $K_D$ of $9 \times 10^{-6}$ M (31) while E-selectin, a receptor mediating tethering of granulocytes to the vascular membrane via recognition of E-selectin-ligand-1, possesses a $K_D$ of $6.2 \times 10^{-5}$ M (32). The Ag-binding sites of anti-carbohydrate Abs, such as those directed against either Chlamydia or Shigella LPS O Ags, possess $K_D$ values ranging from $10^{-5}$ to $10^{-6}$ M (33, 34), while murine anti-carbohydrate Ab responses from conjugate vaccines against meningococcemia yielded $K_D$ estimates ranging from $10^{-6}$ to $10^{-9}$ for Ag (35). The affinity of Dectin-Fc for β-glucan, thus, falls within a range typical for anti-carbohydrate Abs and lectin receptors for their ligands.

Preopsonization of zymosan with Dectin-Fc enhanced baseline macrophage recognition of these particles and this phenotype was lost upon blockade of the primary receptors for murine IgG1, FcγRII, and FcγRIII. Our study demonstrates the sufficiency of the Dectin-1 CRD as a targeting epitope for particulate β-glucan in vitro and is in accordance with evidence suggesting that TLR2 and TLR6, receptors that cooperate with dectin-1 and regulate MyD88- and NF-kB-dependent signaling in response to zymosan, are not required for the actual β-glucan recognition event mediated by Dectin-1 (63). As anticipated, Dectin-Fc bound to PC cysts, demonstrating that the PC cell wall β-glucan is accessible and recognized by this fusion protein. Our observation is in line with studies demonstrating an abundance of PC β-glucan in the inner cyst wall (27) and previous observations that overexpression of the Dectin-1 receptor in RAW macrophages enhanced binding and recognition of PC cysts (16). We have previously shown that Dectin-Fc binds A. fumigatus swollen conidia (53) and our current study now extends the immunolocalization of Dectin-Fc to PC cysts.

Dectin-Fc increased killing of PC by alveolar and elicited peritoneal macrophages and such levels were sustained when macrophage Dectin-1 receptors and were blocked. Apparent efficacy was
lost when FcγRII and FcγRIII were additionally blocked, demonstrating that Dectin-Fc targets PC for degradation through these receptors. The importance of alveolar macrophages in PC degradation and clearance is well-documented (16, 64, 65), yet optimal macrophage-dependent clearance requires coordination with B and T cell-dependent responses against PC, as macrophages and other innate cells are ultimately insufficient in the control of infection. The development of a fusion protein capable of recognizing PC β-glucan coupled with the capacity for FcγRI-dependent targeting represents a novel mechanism for the enhancement of baseline macrophage effector function against PC, in the absence of B and T cell-dependent immune responses against the pathogen.

Adenoviral delivery of Dectin-Fc in SCID mice significantly reduced the kinetics of PC growth and overall PC burden within the lungs by 28 days postchallenge and led to decreased pulmonary LDH and BALF neutrophils, correlates of PC-related lung damage. These findings demonstrate that Dectin-Fc promotes PC recognition and clearance in vivo and increases the specificity of the immune response, presumably through enhancement of FcγRI-dependent clearance mechanisms directed by macrophages. Because the ability of Dectin-Fc to inhibit PC growth is most apparent at late stages of PC infection, as innate host clearance mechanisms lose their capacity to control PC replication within the lungs, our studies suggest that Dectin-Fc may improve the clearance of existing PC lung infections.

Though there was significantly altered growth and an overall reduction of PC burden in SCID mice treated with Dectin-Fc, the establishment of infection could not be prevented. This observation may be a consequence of the expression pattern and relative exposure of β-glucan by PC in the context of the lung environment. Although we have shown that PC β-glucan can be targeted in vitro by macrophages both in this study and in previous work (16), β-glucan may not be present or sufficiently exposed by the as-yet uncharacterized infectious form(s) of the organism, leading to early escape from Dectin-Fc-dependent recognition. In the course of PC infection, soluble β-glucan is shed into the blood and BALF (66) and this may additionally contribute to evasion mechanisms inhibiting β-glucan-dependent recognition of PC organisms. Further, pulmonary collectins target fungal cellular wall carbohydrates and these may compete with Dectin-Fc for binding to PC β-glucan. In particular, surfactant protein D (SP-D) targets β-1,6-linked glucan (67) and recognition of these PC cell wall epitopes by SP-D may inhibit binding of Dectin-Fc to its primary target, β-1,3-linked glucan. Notably, SP-D-deficient mice clear PC more rapidly than wild-type mice (68) and while SP-D is capable of recognizing PC β-glucan coupled with the capacity for FcγRI-dependent targeting represents a novel mechanism for the enhancement of baseline macrophage effector function against PC, in the absence of B and T cell-dependent immune responses against the pathogen.

Models of vaccination against PC have demonstrated that immunity carried by Abs can protect CD4+ T cell-deficient hosts (14) and strategies to develop Ab responses against PC within the actual setting of CD4+ T cell deficiency are emerging (12, 17). Targeting PC β-glucan with Dectin-Fc by passive vaccination, as demonstrated here through adenoviral delivery, may complement these evolving approaches. By targeting PC specifically to FcRs on myeloid cells such as dendritic cells, Dectin-Fc could potentially improve aspects of PC Ag presentation to residual CD4+ T cells. Also, as Dectin-Fc recognizes what is thought to be the most inflammatory component of fungal cell wall (58) and recognition of PC by dectin-1 leads to the elicitation of host chemotactic factors such as MIP-2 (16), this fusion protein could function to limit potentially detrimental host responses to PC pneumonia, such as excessive neutrophil recruitment into the lungs (48).

Our work supports the concept that Ab-dependent targeting of β-glucan may be a promising strategy against PC infection in the immunocompromised host. Continued effort to understand the potential of fungal carbohydrates as targets of protective immunity, or as Ags in the generation of protective immunity, may inform on optimal vaccination strategies against PC pneumonia.

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Disclosures
The authors have no financial conflict of interest.

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